GENOMICS, MOLECULAR GENETICS & BIOTECHNOLOGY

Induction of Somatic Embryogenesis and Plant Regeneration in Select Georgia and Pee Dee Cotton Lines

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ABSTRACT

The current standard strategy for cotton transformation uses *Agrobacterium* for gene transfer and regeneration via somatic embryogenesis, but it is successful only for a handful of cultivars. This study was undertaken with the prospect of expanding the number of elite Upland cotton (*Gossypium hirsutum* L.) genotypes that can be regenerated via somatic embryogenesis. We tested 15 elite Upland cotton lines from Southeast germplasm: eight lines developed by the Georgia Agriculture Experiment Station and seven by the USDA-ARS Pee Dee cotton breeding program. These genotypes were tested on three embryo initiation–maturation media that were previously found to be capable of inducing somatic embryogenesis in diverse cotton species. Three Pee Dee lines, PD 97019, PD 97021, and PD 97100, and one Georgia line, GA 98033, responded to at least one of the three medium treatments. As expected, the regeneration efficiency of the Georgia and Pee Dee lines was relatively low as compared with the standard Coker 312 cultivar and a high degree of seed-to-seed variability was observed. However, the mean number of somatic embryos (SEs) per gram of tissues was high for the two best embryogenic lines, PD 97019 and GA 98033. Furthermore, the percentage of SEs that converted into plantlets for GA 98033 was comparable to Coker 312 for two of the three media tested.

The upland cotton genetic transformation strategy uses *Agrobacterium*-mediated transformation and regeneration via somatic embryogenesis. While *Agrobacterium*-mediated transformation is highly efficient in inserting transgenes into cotton cells (Sunilkumar and Rathore, 2001), the major obstacle lies in the ability to recover transgenic plants from the transformed cells (Wilkins et al., 2000). Most cotton cultivars are recalcitrant to plant regeneration through somatic embryogenesis. The ability to initiate somatic embryos (SEs) appears to be genotype-dependent (Trolinder and Xhixian, 1989), with the most successful cultivars belonging to those released by the Coker Pedigree Seed Co. The lack of embryogenic lines provided the justification for attempting other regeneration approaches in gene transformation (Chlan et al., 1995; Zapata et al., 1999). However, these methods suffer from extremely low transformation efficiencies. Essentially all commercial cultivars in the market were developed by first transforming the transgene into a highly regenerable Coker line, typically from Coker 312, and then introducing the transgene into the elite cultivars by backcross breeding (Trolinder, 1995). The availability of additional cultivars or elite lines that can be directly transformed would greatly benefit the development of transgenic cotton cultivars. Increasing the number of regenerable genotypes could also benefit cotton genomic research by augmenting the use of gene complementation, gene knock-out, or other similar approaches in gene discovery and gene confirmation where the choice of genotype to be transformed is restricted.

Regeneration in *G. hirsutum* was first obtained via spontaneous somatic embryogenesis of cotyledon tissues (Davidonis and Hamilton, 1983). Since then, somatic embryogenesis and regeneration of plants in cotton have been reported by several authors (Trolinder and Goodin, 1987; Trolinder and Xhixian, 1989; Firoozabady and DeBoer, 1993; Sakhanokho et al., 2001). The most significant work was by Trolinder and others, who, in a series of publications, documented that somatic embryogenesis response in cotton was highly genotype specific (Trolinder and Xhixian, 1989), and further described a model regeneration system for *G. hirsutum* based on the highly regenerable Coker 312 cultivar (Trolinder and Goodin, 1987; 1988a; 1988b). Although more than 70 genotypes have now been identified to be capable of differentiating from callus into SEs (Wilkins et al., 2000; Rangan et al., 1984; Cousins et al., 1991; Rangan and Rajasekaran, 1993; Rangan et al., 2003a; 2003b; Mishra et al., 2003), only a few have been successfully regenerated into mature plants. Furthermore, the number of commercial cultivars or elite germplasm lines, with superior fiber and agronomic properties, that can undergo plant regeneration, remains low.

The extent of genetic factors affecting somatic embryogenesis is not well understood, although quantitatively inherited genes have been suggested (Mishra et al., 2003). It is clear, however, that the ability to produce SEs can be transmitted from parents to progenies in the F_1, F_2, and backcross generations (Gawel and Robacker, 1990; Kumar et al., 1998). Therefore, it is of little surprise that Coker 312 and many of its sister lines and derivatives displayed a high embryogenic potential (Trolinder and Xhixian, 1989).

Because Coker cultivars belong to the eastern germplasm type (Niles and Feaster, 1984), there is a possibil-

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ity that other cultivars in this germplasm type, which include those developed by the McNair, Dixie King, Pee Dee, and Georgia Experiment Station breeding programs, could also have somatic embryogenesis capabilities. These breeding programs bred for cultivars adapted to the same production region, with similar growth characteristics and fiber properties. Also, at least in the Pee Dee program, historical and pedigree accounts have indicated that many of the Coker lines were used as parental materials in the cultivar development programs (Culp and Harrell, 1974, p. 1–12; VanEsbroeck et al., 1997). However, with the exception of Coker cultivars, the somatic embryogenic capabilities of most of the eastern type cultivars have not been tested. The objective of this study was to determine the regeneration potential through somatic embryogenesis for a collection of elite breeding lines developed by the Georgia Agricultural Experiment Station and the Pee Dee cotton breeding programs. The improved regeneration protocol of Sakhanokho et al. (2001) that has permitted induction of somatic embryogenesis in a wide range of cotton genotypes from *G. hirsutum*, *G. barbadense* L., and *G. arboreum* L. was used in this study.

MATERIALS AND METHODS

Plant Material

The eight elite Georgia germplasm lines tested were those developed by S. Baker, retired Univ. of Georgia cotton breeder, and lines bred by O.L. May (GA 161, GA 94894, GA 96199, GA 96211, GA 9654, GA 98015, GA 98033, and GA 98084). The seven Pee Dee lines (PD 97006, PD 97019, PD 97021, PD 97047, PD 97072, PD 97100, and PD 97101) from the USDA/ARS, Florence, SC, were bred by O.L. May in the late 1990s. Seeds of Coker 312, the standard cultivar for somatic embryogenesis, were obtained from Dr. K. Rajasekaran, USDA/ARS, New Orleans, LA, and included in the study as a positive control.

Seed Sterilization and Germination

Seeds were placed in 250-mL flasks and surface sterilized by rinsing in 100% ethanol (30 to 60 s) then shaken in 23% commercial bleach [5.25% (v/v) NaOCl] with one drop of Tween 20 (polyoxyethylene-sorbitan monolaurate, Sigma, St. Louis, MO) for 20 min at 110 rpm. Seeds were rinsed three to four times with sterile distilled water and soaked overnight. The next day, the seeds were rinsed again with sterile distilled water to remove any soap and/or bleach residues. Seed coats were removed to enhance germination efficiency (Sakhanokho et al., 2001) before placing the seeds on MS0 medium containing MS salts (Murashige and Skoog, 1962), 30 g L^{-1} glucose, 0.75 mg L^{-1} $MgCl_2$ (pH 5.8), and 2 g L^{-1} Gelrite (Merck & Co., Inc., Rahway, NJ). The medium was sterilized by autoclaving for 20 min at 121°C. Seeds were placed into 100- by 15-mm Petri dishes containing about 40 mL of media and cultured in a growth chamber maintained at 28 ± 2°C and under a 16/8 h (day/night) light regime.

Callus Initiation/Proliferation and Cell Suspension Cultures

The callus initiation and the liquid suspension culture treatment steps were identical for all genotypes. Explants used for callus initiation consisted of 4- to 10-d-old hypocotyls and cotyledons. Hypocotyl segments (5-mm sections) were split longitudinally and each cotyledon was cut into seven to eight pieces. These hypocotyl and cotyledon sections were transferred to 100- by 20-mm Petri dishes containing callus induction medium (CIM), which was made up of MS salts supplemented with 2.0 mg L^{-1} NAA, 1.0 mg L^{-1} kinetin, 0.4 mg L^{-1} thiamine, 100 mg L^{-1} myo-inositol, 30 g L^{-1} glucose, and 0.75 g L^{-1} $MgCl_2$ (pH 5.8). The gelling agent (2 g L^{-1} Gelrite) was added to the medium before autoclaving it at 121°C for 20 min. Most explants, especially the hypocotyl explants, formed callus after about 4 wk. Pre-embryogenic callus was selected on the basis of the morphology and characteristics as previously described (Firoozabady and DeBoer, 1993; Sakhanokho et al., 2001). The prolific, loose embryogenic callus, with small cells that contained very dense cytoplasm, was selected and then transferred onto fresh CIM medium until an adequate amount of callus (1–3 g) was obtained. Friable embryogenic callus was transferred into 125-mL transparent polymethylpentene jars (Nalgene, Suwanee, GA) containing 70 mL of embryo induction medium (liquid) or EIML (Sakhanokho et al., 2001), which was sterilized by autoclaving for 15 min at 121°C. Briefly, the EIML medium consisted of MS salts in which NH_4NO_3 was removed and the amount of KNO_3 was doubled and supplemented with 10 mg L^{-1} thiamine, 100 mg L^{-1} myo-inositol, 1 mg L^{-1} nicotinic acid, 1 mg L^{-1} pyridoxine, 1 g L^{-1} glutamine, 0.5 g L^{-1} asparagine, 30 g L^{-1} glucose (pH 5.8). The jars were shaken at 130 rpm under a 16/8 h light/dark cycle at 28°C for a period of 4 to 6 wk.

Embryo Development/Maturation and Rooting Media

After 4 to 6 wk, cell suspension cultures were fractionated through a tea strainer with 40-mesh screens pore size. Callus aggregates that did not pass through the mesh screens and contained embryogenic cells were weighed and placed on development/maturation media, S15 g.05NAA, $EMMS_2$, or $EMMS_4$. The medium S15 g.05NAA consisted of MS salts supplemented with 0.05 mg L^{-1} NAA, 15 g L^{-1} sucrose instead of glucose as the carbon source, 2 g L^{-1} Gelrite, and 0.75 g L^{-1} $MgCl_2$ (pH 5.8). $EMMS_2$ medium was similar to EIML medium, but it also contained 0.05 mg L^{-1} NAA, 0.05 mg L^{-1} kinetin, 2 g L^{-1} Gelrite, and 0.75 g L^{-1} $MgCl_2$ (pH 5.8). $EMMS_4$ is similar to $EMMS_2$ except that for growth regulators, $EMMS_4$ contains 0.1 mg L^{-1} 2, 4-D and 0.5 mg L^{-1} kinetin. The weight of the callus transferred was subsequently used to quantify SE production per gram of callus. Cultures were transferred onto fresh media every 4 wk. Germinated SEs with or without well-developed root systems were transferred to Magenta or Nalgene transparent polymethylpentene jars containing the rooting medium (RM), which consisted of MS salts, 0.05 mg L^{-1} IAA, 15 g L^{-1} sucrose, 2 g L^{-1} Gelrite, and 0.75 g L^{-1} $MgCl_2$ (pH 5.8) for further development. Plants were then transferred into pots containing vermiculite or a vermiculite and sphagnum moss mixture and covered with plastic bags, which were punctured with several small holes to allow for gradual acclimatization to ambient humidity. These plastic bags were removed after 1 to 3 wk, depending on plantlet vigor.

The percentage of seedling response within a genotype was determined by counting the number of seedlings that produced SEs compared to the total number of seedlings tested. The fraction of SEs germinated into plantlets in one gram of plated suspension cells was calculated and expressed as the percentage of plantlets over the total number of embryos in each genotype by media combination. The experimental design was a randomized complete block design with 16 genotypes and

three types of media. Each experimental unit consisted of 10 seeds per genotype. In addition, each seedling was tested in five plates (two for hypocotyl explants and three for cotyledon explants) as subsamples. The experiment was replicated six times. The analysis of variance and mean calculations were performed by SAS program systems for Windows, version 8.2 (SAS Inst., Inc. 2001).

RESULTS AND DISCUSSION

Effect of Explant Source on Callus and Embryo Initiation

All genotypes tested produced callus on CIM medium within 2 to 4 wk. The hypocotyl explants formed callus more readily than explants from cotyledons, a result that agrees with earlier findings (Trolinder and Goodin, 1988a; Sakhanokho et al., 2001). The cotyledon tissues, particularly from the older explants, have the propensity for excessive root formation (Fig. 1A), resulting in less callus formation. Following callus proliferation, a liquid culture step was added to allow maximum medium-to-tissue contact, which promotes rapid SE initiation in many plant species, including cotton (Jones and Petolino, 1988; Finer, 1988; Gawel and Robacker 1990; Samoylov et al., 1998; Sakhanokho et al., 2001). At the end of a 4- to 6-wk period in suspension culture, some cell clusters from Coker 312 began to form embryo-like structures and even SEs. However, no SEs were observed in the Georgia or Pee Dee lines at this stage. Generally, SE development for Coker 312 occurred during the liquid suspension step or 1 to 4 wk after plating in embryo development–maturation media (Fig. 1B). The suspension cells from Georgia and Pee Dee lines required 7 wk or more of culture in the embryo development–maturation media before SE development was observed. More SEs were consistently induced from hypocotyl callus than cotyledon explants for all media types tested (Fig. 2). Furthermore, the more rapid callus development from the hypocotyl explants may also shorten the culture duration, thus reducing the occurrence of somaclonal variation, a major problem in cotton tissue culture.

Genotype and Seed-to-Seed Variation

Not all seeds in an embryogenic line produce SEs, and specific individuals within a cultivar may be more embryogenic than others (Trolinder and Xhixian, 1989). In this study, a minimum of 60 seedlings were tested for each genotype, thus providing a good survey on the level of heterogeneity in terms of the number of seedlings that could produce SE. The control Coker 312 showed a high frequency of embryogenesis, with 92 to 100% of seedling explants exhibiting somatic embryogenesis in the three media treatments (Fig. 3). Among the Georgia and Pee Dee lines, the genotypes PD 97019, PD 97021, PD 97100, and GA 98033 were found to be embryogenic (Table 1). The genotypes PD 97019 and GA 98033 produced SEs on all embryo initiation/development media, with frequencies ranging from 8 to 15% (Fig. 3). SE induction in the two least embryogenic lines, PD 97021 and PD 97100, appeared to be media dependent. The former genotype produced SEs from only 5% of the seedlings on S15.05NAA and EMMS2 media while 3% of PD 97100 seedlings tested exhibited somatic embryogenesis on only the S15 g.05NAA medium (Fig. 3).

The low percentage of seedlings in the Georgia and Pee Dee lines that produced SEs was not a surprise since extensive seed-to-seed variability in embryogenic capability was observed in almost all cotton cultivars tested, including Coker 312 when commercial seed lots were used (Trolinder and Xhixian 1989; Gawel and Robacker, 1990; Sakhanokho et al., 2001; Mishra et al., 2003). Although factors such as tissue and media types, as well as microenvironment conditions in tissue culture may affect embryo formation, the seed-to-seed variability in embryogenic capability could also have originated during the cultivar development process where different F4 or F5 plants were bulked (O.L. May, personal communication), leaving a considerable amount of genetic heterozygosity and heterogeneity for traits, such as somatic embryogenesis, that were not under any selection pressure (Kumar et al., 1998; Mishra et al., 2003). At least two lines of evidence are available that support this conjecture. First, an improvement in somatic embryogenesis frequency was achieved in Coker 310 (Kumar et al., 1998) and Acala Maxxa (Mishra et al., 2003) cultivars by selecting for embryogenic lines within the cultivar. In fact, the embryogenic response of Coker 312 observed in this study was significantly higher than previously reported because the seed source of this line was specifically selected for a high regeneration poten-

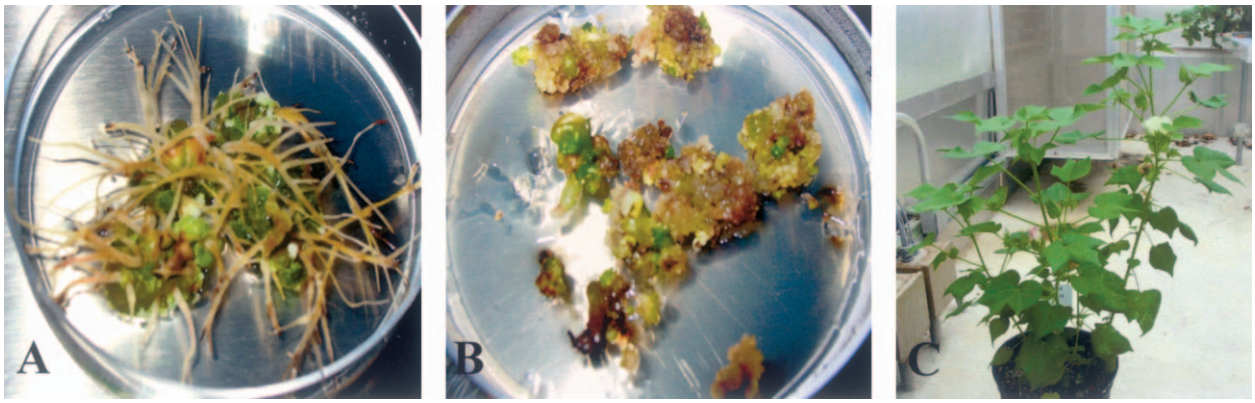

Fig. 1. Somatic embryogenesis and plant regeneration of Pee Dee and Georgia lines. (A) Extensive roots formation from cotyledon-derived callus. (B) Embryogenic cultures. (C) Mature plant regenerated from GA 98033.

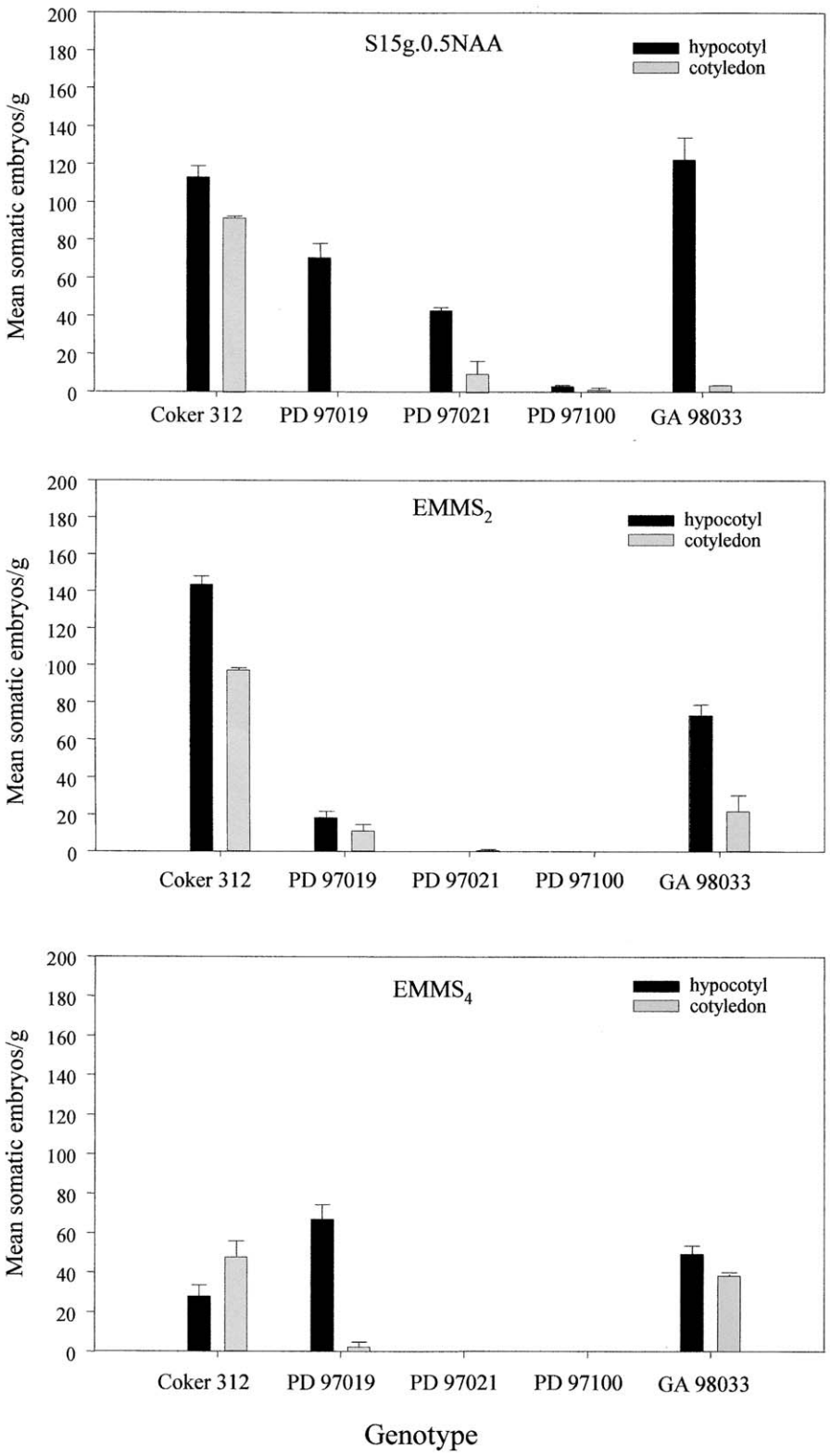

Fig. 2. Effect of source of explant on the average number of somatic embryos (SEs) induced from calluses of five cotton genotypes cultured on three embryo initiation or germination media.

tial (K. Rajasekaran, personal communication). Second, genetic studies on cotton embryogenesis have shown that the ability to produce SEs could be inherited from parents to progenies in intraspecific (Gawel and Robacker, 1990; Kumar et al., 1998) and interspecific crosses (Sakhanokho and Chee, unpublished data); hence, Coker 312 and many of its sister lines and derivatives maintained a high embryogenic potential (Trolinder and

Table 1. The percentage of somatic embryos (SEs) that germinated into plantlets for five cotton lines cultured on three embryo initiation/maturation media.

	Percentage of SEs converted to plantlets†				
Medium	**Coker 312**	**PD 97019**	**PD 97021**	**PD 97100**	**GA 98033**
S15g.05NAA	**42.3 + 2.1**	**23.6 + 8.2**	**13.6 + 3.1**	**11.6 + 5.6**	**41.3 + 4.2**
$EMMS_2$	**33.5 + 9.3**	**26.4 + 8.2**	**13.6 + 2.8**	**0 + 0**	**43.8 + 3.3**
$EMMS_4$	**66.5 + 5.2**	**38.4 + 6.1**	**0 + 0**	**0 + 0**	**50.1 + 2.3**

† Values represent the percentage ± SD of plantlets over the total number of embryos in one gram of embryogenic callus. Each treatment consisted of the mean of 60 explants.

Xhixian, 1989). Since Coker and Pee Dee belong to the eastern germplasm group, and many of the Coker lines have appeared in the pedigree of many Pee Dee lines (Culp and Harrell, 1974, p. 1–12), it is therefore quite possible that the genes conferring somatic embryogenesis could persist in the modern Pee Dee germplasm.

Effect of Media on Embryo Initiation, Maturation, and Conversion

Although the frequency of seedlings that gave rise to SEs was low within the regenerable Georgia and Pee Dee lines, the number of SEs produced from the regenerable plants was high for the two best embryogenic lines, PD 97019 and GA 98033. For example, the mean SEs induced per gram of initial callus for GA 98033 was comparable to that of Coker 312 on the S15 g.05NAA medium (Fig. 4). Similarly, both PD 97019 and GA 98033 showed higher SE induction than Coker 312 on the EMMS4 medium. However, with the exception of PD 97019, most lines performed poorly on this medium. The genotypes PD 97100 and PD 97021, which showed SE induction only on selected media, performed poorly by producing very few SEs per grams of callus.

The success rate in converting SEs into plantlets was generally very low as many SEs did not germinate or undergo normal developmental processes to survive into plants. In this study, only germinated SEs with well-developed root systems and two well-developed cotyledons were counted as plantlets. For GA 98033, the percentage of SEs converted into plantlets was at least comparable to Coker 312 in two of the three media tested. The percentage of SEs that converted to plantlets for the other three regenerable Pee Dee lines was lower than for Coker 312 (Table 1). Several authors have cited the establishment of cultured plants with good root systems as one of the major hurdles often encountered in cotton tissue culture (Gould et al., 1991; Zapata et al., 1999). This problem appears to be even more acute in *G. arboreum* (Sakhanokho et al., 2000), a diploid progenitor of tetraploid cotton and considered by many to be a model species for mapping complex traits and for gene cloning. However, in this study, rooting was not a problem per se in any of the genotypes tested, although several other problems inherent to cotton tissue culture were observed, including the formation of abnormal SEs with fused or unopened cotyledons.

The acclimatization of plantlets from in vitro environments to ex vitro conditions was relatively easy for Coker 312 and GA 98033 plantlets, requiring at times an acclimatization period as short as 1 to 2 wk before the complete removal of the protective plastic bags covering the plantlets. On the other hand, this step took 4 wk or more for Pee Dee lines. Regenerated plantlets from these lines, though appearing as normal as those from Coker 312 and GA 98033 in Magenta jars, were very sensitive to ex vitro conditions. This led, on transfer to potting soil in the greenhouse, to the loss of 80% (16/20), 86% (42/49), and 100% (5/5) of the plantlets from PD 97021, PD 97019, and PD 97100, respectively. In addition, the longer culturing and acclimatization time, which can take from 7 to 9 mo for these Pee Dee lines, is a concern as this often creates an environment favorable to somaclonal variation. For example, only one of four mature plants from PD 97021 and four of seven from PD 97019 are setting bolls, with the rest exhibiting sterility. In contrast, only minimal abnormalities were observed in GA 98033. With the exception of six (13%) sterile plants, all 39 (87%) plants grew vigorously in the greenhouse and produced viable seeds. No other apparent morphological abnormalities were observed in these plants (Fig. 1C).

Expanding the List of Embryogenic Cotton Lines

The cotton industry has benefited tremendously from transgenic cultivars, yet cotton is one of the most recalcitrant species to tissue culture and plant regeneration. The addition of these Georgia and Pee Dee lines to the short list of regenerable cotton genotypes is significant

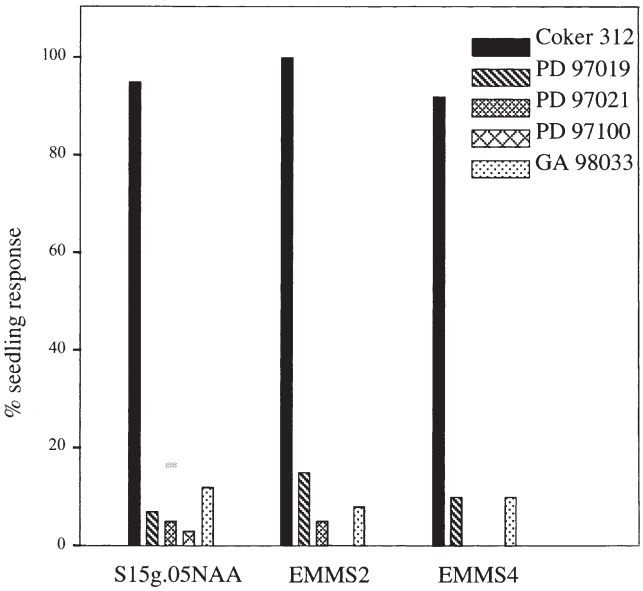

Fig. 3. Comparison of genotypes for the percentage of seedlings (explants) that gave rise to SEs when cultured on three embryo initiation–germination media.

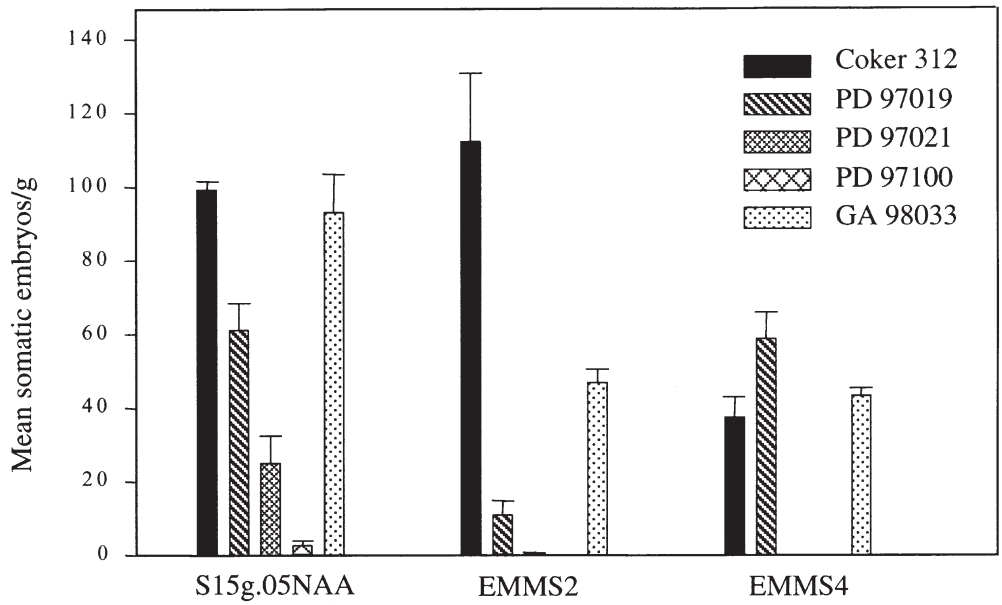

Fig. 4. Mean number of somatic embryos (SEs) induced from calluses of five cotton genotypes cultured on three embryo initiation or germination media.

to the cotton germplasm in two different ways. First, the Georgia and Pee Dee lines were developed by cotton breeders at publicly funded programs. Thus, these embryogenic Georgia and Pee Dee lines have no restrictions of use like those imposed on cultivars developed by private cotton breeding companies. Second, the embryogenic GA98033 represents a new germplasm line that combines high yield potential, acceptable fiber quality, and resistance to Fusarium wilt (caused by *Fusarium oxysporum* Schlechtend). In the 2002 University of Georgia dryland late maturity cotton variety trial, GA 98033 had an acceptable fiber length and micronaire, superior strength, and most importantly, ranked number five in yielding ability in four combined locations (Day et al., 2003). In that trial, it outperformed many of the leading commercial cultivars, including many transgenics that are currently in production.

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